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November 22, 2000

Assistant Commissioner for Patents
Washington, DC 20231

SUBMISSION OF PRIORITY DOCUMENTS

Sir:

It is respectfully requested that this application be given the benefit of the foreign filing date under the provisions of 35 U.S.C. §119 of the following, a certified copy of which is submitted herewith:

Application No.	Country of Origin	Filed
98870087.8	EP	17 APRIL 1998

Respectfully submitted,

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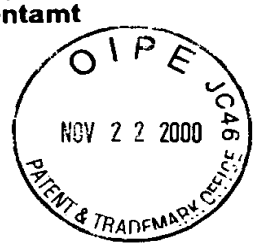

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98870087.8

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
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Blatt 2 d r B scheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Improved immunodiagnostic assays by using reducing agents

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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See for original title of the application page 1 of the description

Methods for improving the conformation of proteins by means of reducing agents

The present invention relates to the field of diagnosis and treatment of HCV infection. More particularly, the present invention relates to HCV NS3 helicase and its uses. Also the present invention relates to improved immunodiagnostic assays.

Background of the invention

Hepatitis C Viruses (HCV) constitute a genus within the Flaviviridae, with closest homology to the hepatitis G and GB viruses, and Pestiviruses. The positive-stranded RNA genome encodes at least 9 proteins. Core, E1, and E2 constitute the structural proteins; NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural (NS) proteins. HCV isolates display high levels of sequence heterogeneity allowing classification into at least 11 types and 90 subtypes (Maertens and Stuyver, 1997). HCV infection of the human liver is often clinically benign, with mild icterus in the acute phase; the disease may even go unnoticed in some cases of acute resolving hepatitis C. In the majority (>70%) of cases, however, HCV infection leads to chronic persistent or active infection, often with complications of liver cirrhosis and auto-immune disorders. Hepatocellular carcinoma may occur after about 20 to 35 years (Saito et al., 1990); sometimes even without the intermediate phase of cirrhosis. No prophylaxis is available today and treatment with interferon-alpha (IFN-α) only leads to long-term resolution in about 4 to 36% of treated cases, depending on the HCV genotype (Maertens and Stuyver, 1997).

Since productive culture methods for HCV are currently not available, and since only minute amounts of HCV antigens circulate in the infected patient, direct detection of HCV particles cannot be performed routinely, and indirect diagnosis is only possible using cumbersome amplification techniques for HCV RNA detection. Unlike with many other viral infections, HCV particles generally persist in the blood, liver, and lymphocytes despite the presence of cellular and humoral immune response to most of the HCV proteins. HCV antibodies can be conveniently detected by Elisa techniques which allow high throughput screening in blood banks and clinical laboratories. Supplementary antibody testing is required and is now mandatory in most countries. True HCV reactivity is thus discriminated from false reactivity, which may be caused by non-specific binding of serum or plasma immunoglobulines or anti-idiotypic

components to coating or blocking reagents, to contaminants present in HCV antigen preparations, or even to fusion parts or non-specific regions of the recombinant antigens themselves (McFarlane et al., 1990). HCV RNA detection by PCR or bDNA techniques have recently been introduced to monitor chronic HCV disease, especially during therapy. Surprisingly, HCV RNA detection is sometimes employed to confirm HCV Ab screening tests, despite the fact that only ~70-94% of repeatedly HCV Ab positive patient samples are positive by nested PCR (Marin et al., 1994). Of HCV Ab positive blood donors, who usually present with milder forms of the disease and low HCV RNA levels, confirmation by nested PCR is usually in the order of ~40% (Waumans et al., 1993; Stuyver et al., 1996). Strip-based assays therefore provide the only reliable alternative for HCV Ab confirmation. Even in the case of an indeterminate result in the confirmatory assay, serological follow up of the patient rather than HCV RNA detection is advisable. Since native HCV antigens are not available, such confirmatory assays incorporate synthetic peptides and/or recombinant fragments of HCV proteins. One of the most critical issues in the confirmation of antibodies constitutes the reactivity of the NS3 protein (Zaaijer et al., 1993). NS3 antibodies often appear first in seroconversion series and the reactivity of the NS3 protein seems to be different in the different commercial assays available today .

Innogenetics introduced the concept of strip technology in which usually a combination of synthetic peptides and recombinant proteins are applied as discrete lines in an ordered and easily readable fashion. The INNO-LIA HIV Ab tests have proven to be superior to routinely used western blots (Pollet et al., 1990). The Line Immuno Assay allows multiparameter testing and thus enables incorporation of cutoff and other rating systems, sample addition control, as well as testing for false reactivity to non-HCV proteins used as carrier or fusion partner required for some antigens in the Elisa test. In principle, the test format allows to combine antigens of different aetiological agents or phenotypically linked conditions into a single test.

The INNO-LIA HCV Ab III is a 3rd generation Line Immuno Assay which incorporates HCV antigens derived from the Core region, the E2 hypervariable region (HVR), the NS3 helicase region, and the NS4A, NS4B, and NS5A regions. Table I gives an overview of the polyprotein positions of HCV antigens in 1st, 2nd, and 3rd generation INNO-LIA HCV Ab tests. The first generation test was launched in 1991 and already incorporated Core and NS5 peptides in addition to NS4 antigens. In the second generation test, sensitivity was considerably improved

by introducing biotin-coupled peptides. In the third generation assay, highly purified recombinant subtype 1b NS3 protein and E2 peptides enabled superior sensitivity while safeguarding the reliable specificity which is characteristic of peptide-based tests (Peeters et al., 1993). Perhaps one of the most important features of this assay is its unprecedented correlation with HCV RNA positivity (Claeys et al., 1992; De Beenhouwer et al., 1992; .

The antigens were coated as 6 discrete lines on a nylon strip with plastic backing. In addition, four control lines are coated on each strip: anti-streptavidin, 3+ positive control (anti-human Ig), 1+ positive control (human IgG), and the ± cutoff line (human IgG). A diluted test sample is incubated in a trough together with the LIA III strip. If present in the sample, HCV antibodies will bind to the HCV antigen lines on the strip. Subsequently, an affinity-purified alkaline phosphatase labelled goat anti-human IgG (H+L) conjugate is added and reacts with specific HCV antigen/antibody complexes if previously formed. Incubation with enzyme substrate produces a chestnut-like color, the intensity of which is proportionate to the amount of HCV-specific antibody captured from the sample on any given line. Color development is stopped with sulphuric acid. If no HCV-specific antibodies are present, the conjugate only binds to the ±, 1+, and 3+ control lines. If the addition of sample is omitted, only the ± and 1+ control lines will be stained.

Definitions

The following definitions serve to illustrate the different terms and expressions used in the present invention.

The term 'HCV NS3 protein' refers to a polypeptide or an analogue thereof (e.g. mimotopes) comprising an amino acid sequence (and/or amino acid analogues) defining at least one HCV epitope of either HCV NS3 protease or helicase.

The term 'hepatitis C virus envelope protein' refers to a polypeptide or an analogue thereof (e.g. mimotopes) comprising an amino acid sequence (and/or amino acid analogues) defining at least one HCV epitope of either the E1 or the E2 region (see WO 96/04385 of which the contents are hereby incorporated by reference).

It should also be understood that the isolates used in the examples section of the present invention were not intended to limit the scope of the invention and that any HCV

isolate from type 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or any other new genotype of HCV is a suitable source of HCV sequence for the practice of the present invention.

The HCV antigens used in the present invention may be full-length viral proteins, substantially full-length versions thereof, or functional fragments thereof (e.g. fragments which are not missing sequence essential to the formation or retention of an epitope). Furthermore, the HCV antigens of the present invention can also include other sequences that do not block or prevent the formation of the conformational epitope of interest. The presence or absence of a conformational epitope can be readily determined though screening the antigen of interest with an antibody (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any). In such screening using polyclonal antibodies, it may be advantageous to adsorb the polyclonal serum first with the denatured antigen and see if it retains antibodies to the antigen of interest.

The term 'fusion polypeptide' intends a polypeptide in which the antigen(s), in particularly HCV antigen(s), are part of a single continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or be separated by intervening amino acid sequences. The fusion polypeptides may also contain amino acid sequences exogenous to HCV.

The term 'solid phase' or 'solid support' means a solid body to which the individual HCV antigens or the fusion polypeptide comprised of HCV antigens are bound covalently or by noncovalent means such as by hydrophobic adsorption.***

The term 'biological sample' intends a fluid or tissue of a mammalian individual (e.g. an anthropoid, a human) that commonly contains antibodies produced by the individual, more particularly antibodies against HCV. The fluid or tissue may also contain HCV antigen. Such components are known in the art and include, without limitation, blood, plasma, serum, urine, spinal fluid, lymph fluid, secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk, white blood cells and myelomas. Body components include biological liquids. The term 'biological liquid' refers to a fluid obtained from an organism. Some biological fluids are used as a source of other products, such as clotting factors (e.g. Factor VIII;C), serum albumin, growth hormone and the like. In such cases, it is important that the source of biological fluid be free of contamination by virus such as HCV.

The term 'immunologically reactive' means that the antigen in question will react specifically with anti-HCV antibodies present in a body component from an HCV infected individual.

The term 'immune complex' intends the combination formed when an antibody binds to an epitope on an antigen.

The terms E1 and E2 as used herein are fully described in WO 96/04385 of which the content is incorporated by reference in the present description.

The term 'purified' as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, withouth affecting the determination of the percentage purity as used herein. An 'isolated' HCV protein intends an HCV protein composition that is at least 35% pure.

The term 'essentially purified proteins' refers to proteins purified such that they can be used for in vitro diagnostic methods and as a therapeutic compound. These proteins are substantially free from cellular proteins, vector-derived proteins or other HCV viral components. Usually these proteins are purified to homogeneity (at least 80% pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%, and most preferably the contaminating proteins should be undetectable by conventional methods like SDS-PAGE and silver staining.

The term 'recombinantly expressed' used within the context of the present invention refers to the fact that the proteins of the present invention are produced by recombinant expression methods be it in prokaryotes, or lower or higher eukaryotes as discussed in detail below.

The term 'lower eukaryote' refers to host cells such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular. Preferred lower eukaryotes are yeasts, particularly species within Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia (e.g. Pichia pastoris), Hansenula (e.g. Hansenula polymorpha), Yarowia,

Schwaniomyces, Schizosaccharomyces, Zygosaccharomyces and the like. Saccharomyces cerevisiae, S. carlsbergensis and K. lactis are the most commonly used yeast hosts, and are convenient fungal hosts.

The term 'prokaryotes' refers to hosts such as E.coli, Lactobacillus, Lactococcus, Salmonella, Streptococcus, Bacillus subtilis or Streptomyces. Also these hosts are contemplated within the present invention.

The term 'higher eukaryote' refers to host cells derived from higher animals, such as mammals, reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells), baby hamster kidney (BHK), pig kidney (PK15), rabbit kidney 13 cells (RK13), the human osteosarcoma cell line 143 B, the human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. Spodoptera frugiperda). The host cells may be provided in suspension or flask cultures, tissue cultures, organ cultures and the like. Alternatively the host cells may also be transgenic animals.

The term 'polypeptide' refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term 'recombinant polynucleotide or nucleic acid' intends a polynucleotide or nucleic acid of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation : (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

The term 'recombinant host cells', 'host cells', 'cells', 'cell lines', 'cell cultures', and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original

cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

The term 'replicon' is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

The term 'vector' is a replicon further comprising sequences providing replication and/or expression of a desired open reading frame.

The term 'control sequence' refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term 'control sequences' is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

The term 'promoter' is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent structural gene.

The expression 'operably linked' refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence 'operably linked' to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An 'open reading frame' (ORF) is a region of a polynucleotide sequence which encodes a polypeptide and does not contain stop codons; this region may represent a portion of a coding sequence or a total coding sequence.

A 'coding sequence' is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can

include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

As used herein, 'epitope' or 'antigenic determinant' means an amino acid sequence that is immunoreactive. Generally an epitope consists of at least 3 to 4 amino acids, and more usually, consists of at least 5 or 6 amino acids, sometimes the epitope consists of about 7 to 8, or even about 10 amino acids. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof. Such equivalents also include strain, subtype (=genotype), or type(group)-specific variants, e.g. of the currently known sequences or strains belonging to genotypes 1a, 1b, 1c, 1d, 1e, 1f, 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 4h, 4i, 4j, 4k, 4l, 5a, 5b, 6a, 6b, 6c, 7a, 7b, 7c, 8a, 8b, 9a, 9b, 10a, or any other newly defined HCV (sub)type. It is to be understood that the amino acids constituting the epitope need not be part of a linear sequence, but may be interspersed by any number of amino acids, thus forming a conformational epitope.

The term 'immunogenic' refers to the ability of a substance to cause a humoral and/or cellular response, whether alone or when linked to a carrier, in the presence or absence of an adjuvant. 'Neutralization' refers to an immune response that blocks the infectivity, either partially or fully, of an infectious agent. A 'vaccine' is an immunogenic composition capable of eliciting protection against HCV, whether partial or complete. A vaccine may also be useful for treatment of an individual, in which case it is called a therapeutic vaccine.

The term 'therapeutic' refers to a composition capable of treating HCV infection.

The term 'effective amount' refers to an amount of epitope-bearing polypeptide sufficient to induce an immunogenic response in the individual to which it is administered, or to otherwise detectably immunoreact in its intended system (e.g., immunoassay). Preferably, the effective amount is sufficient to effect treatment, as defined above. The exact amount necessary will vary according to the application. For vaccine applications or for the generation of polyclonal antiserum / antibodies, for example, the effective amount may vary depending on the species, age, and general condition of the individual, the severity of the condition being treated, the particular polypeptide selected and its mode of administration, etc. It is also believed that effective amounts will be found within a relatively large, non-critical range. An appropriate effective amount can be readily determined using only routine experimentation.

Preferred ranges of E1 and/or E2 and/or E1/E2 single or specific oligomeric envelope proteins for prophylaxis of HCV disease are 0.01 to 100 μg/dose, preferably 0.1 to 50 μg/dose. Several doses may be needed per individual in order to achieve a sufficient immune response and subsequent protection against HCV disease.
Preferred ranges of NS3 proteins for prophylaxis of HCV disease are ....

Aims of the invention

It is an aim of the present invention to provide improved HCV diagnostic assay components.

More particularly it is an aim of the present invention to provide improved HCV NS3 protein preparations for use in HCV antibody diagnosis and/or HCV treatment.

It is further an aim of the present invention to provide a method for increasing the reactivity of HCV antibodies with recombinant NS3 helicase protein present on a solid phase.

It is also an aim of the present invention to provide a novel method for purifying cysteine containing recombinant proteins, more particularly recombinant HCV proteins.

It is also an aim of the present invention to provide new HCV NS3 protein encoding sequences.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

Detailed description of the invention

The present invention relates more particularly to a solid phase immunoassay comprising on said solid phase an antigen in the presence of a reducing agent. As is demonstrated in the Examples section the present inventors have found that the presence of a reducing agent such as DTT besides an antigen coated to a solid phase renders a solid phase immunassay coupled antigen much more reactive with antibodies directed to said antigen.

A reducing agent according to the present invention is any agent which achieves reduction of S-S disulphide bridges. Reduction of the 'S-S' disulphide bridges is a chemical reaction wherby the disulfides are reduced to thi l (-SH). The disuphide bridges breaking

agents and methods disclosed in WO 96/04385 are hereby incorporated by reference in the present description. 'S-S' Reduction can be obtained by (1) enzymatic cascade pathways or by (2) reducing compounds. Enzymes like thioredoxin, glutaredoxin are known to be involved in the in vivo reduction of disulfides and have also been shown to be effective in reducing 'S-S' bridges in vitro. Disulfide bonds are rapidly cleaved by reduced thioredoxin at pH 7.0, with an apparent second order rate that is around 10^4 times larger than the corresponding rate constant for the reaction with DTT. The reduction kinetic can be dramatically increased by preincubation the protein solution with 1 mM DTT or dihydrolipoamide (Holmgren, JBC, 1979).

Thiol compounds able to reduce protein disulfide bridges are for instance Dithiothreitol (DTT), Dithioerythritol (DTE), 2-mercaptoethanol, thiocarbamate, bis (2-mercaptoethyl)sulfone and N,N'-bis(mercaptoacetyl)hydrazine , sodium-dithionite.

Reducing agents without thiol groups like ascorbate, stannous cloride ($SnCl_2$) which have been shown to be very useful in the reduction of disulfide bridges in monoclonal antibodies (Nucl. Med. Biol., 18, 227- 233, 1991) may also be used for the NS3 reduction. Sodium borohydride treatment has been shown to be effecftive for the reduction of disulfide bridges in peptides (Anal. Biochem., 214, 334, 1993) .Tris (2-carboxyethyl)phosphine (TCEP) is able to reduce disulfides at low pH (Burns et al., J.org. Chem., 56, 2648, 1991) . Selenol catalyses the reduction of disulfide to thiols when DTT or sodium borohydride is used as reductant. Selenocysteamine, a commercially available diselenide, was used as precursos of the catalyst (Singh & Kats, Anal. Biochem.,232, 86-91, 1995).

The present invention relates more particularly to a method for producing an immunoassay as defined above wherein said reducing agent is added to said solid phase during the steps of coating, blocking and/or fixation of said antigen to said solid phase.

Blocking can occur via any method known in the art can for instance also be performed using albumine, serum proteins or polyvinylpyrolidone or detergents

Coating conditions can vary widely as known by the skilled person and involves applying to a solid phase and allowing a reaction to occur. Different buffers known by the skilled man may be used for this step.

Fixation can occur according to any method known in the art.

Examples of blocking, fixation and coating conditions are given in the Examples

section.

The present invention relates even more particularly to a method as defined above wherein said reducing agent is added to said solid phase during the step of coating the antigen to the solid phase. Examples of coating buffers are given in the Examples section. All other known coating buffers known in the art also form part of the present disclosure.

The present invention relates also to a method as defined above, wherein said reducing agent is added to said solid phase during the step of blocking said solid phase comprising the antigen applied thereto in the presence of a blocking agent. Examples of blocking buffers are given in the Examples section. All other known blocking buffers known in the art also form part of the present disclosure.

The present invention relates also to a method as defined above, wherein said reducing agent is added to said solid phase during the step of fixation of said solid phase comprising the antigen applied thereto in the presence of a fixating agent. Examples of fixation buffers are given in the Examples section. All other known fixation buffers known in the art also form part of the present disclosure.

The present invention relates preferably to a method as defined above wherein said reducing agent is DTT or DTE.

The present invention relates also to a method according to any of claims 1 to 5 wherein said reducing agent is used in a concentration range of 0.1 mM to 1 M, more particularly from 0.5 mM to 500 mM, even more particularly from 1 mM to 250 mM, most partcularly from 1 to 50 mM. Some applications may require ranges from 0.5 to 50 mM, 1 to 30 mM, 2 to 20 mM, 5 to 15 mM, or about 10 mM reducing agent. DTT is particularly preferred as a reducing agent.

The present invention also relates to a method as defined above wherein said antigen is an HCV NS3 protein. More particularly an HCV NS3 helicase. Also preferred is an HCV envelope protein such as E1 and/or E2 protein. Also any other protein known in the art may react better with antibodies against said protein when the protein is added to the solid phase in the presence of DTT.

The present invention also relates to a solid phase immunoassay produced by a method as defined above.

The present invention also relates to an ELISA produced by a method according to

claim 2 wherein said reducing agent is preferably added in the coating and fixation steps.

The present invention preferably relates to a Line Immunoassay produced by a method according to claim 2 wherein said reducing agent is preferably added in the blocking step.

The present invention also relates to the use of an assay as defined above for in vitro diagnosis of antibodies raised against an antigen as defined above.

The present invention also relates to an HCV NS3 protein treated by a method comprising the steps of sulphonation and subsequent desulphonation.

Sulphonation and desulphonation is a reaction whereby $-SO_3$ groups are introduced or removed respectively from the protein. Sulphonation is defined as a process where thiols and disulphide bonds are converted to S-Sulphonates, according to reactions:

$RSH \longrightarrow RS\text{-}SO_3^-$ (1)

$RS\text{-}SR + 2\ \text{-}SO_3^- + H_2O \longrightarrow 2\ RS\text{-}SO_3^- + 2\ OH^-$ (2)

The product of the reactions are S-Sulphoproteins and are normally stable under neutral pH conditions . Reaction (1) can be obtained by incubation the protein solution with tetrathionate at pH >7 (Inglis &Liu, 1970). Reaction (2) proceeds to completion in the presence of Cupric ions (Cole, 1967). Chan (Biochemistry, 7, 4247-4253, 1968) has treated the protein with sodium sulfite and catalytic amounts of cysteine in the presence of oxygen to obtain sulfo-proteins. Kella & Kinsella have described a controlled cleavage of disulfide bonds in proteins by sulfonation in the absence of denaturants, and this phenomenon was explained by the fact that not all 'S-S' bridges have the same accesibility. On the other hand they showed that the sulfitolysis kinetics depended on the used denaturant (Int. J. Peptide Protein Res., 28, 586-592, 1986).

Desulfonation can be obtained (1) by an excess of competitive -SH (thiol) groups, (2) by reducing agents or (3) by incubation in non- neutral pH conditions.

$RS\text{-}SO_3^- + R'SH \longrightarrow RSH + R'S\text{-}SO_3^-$

$RS\text{-}SO^-_3 +$ reductans $\longrightarrow RSH$

Competitive thiol groups may be (1) from low Mr-compounds or (2) from proteinaeous -SH groups.

As mono r dithiol containing compounds may be cited:

cysteine, cysteamine, reduced gluthation,N -acetyl cysteine, homocysteine mercaptoethanol, thiocarbamates,bis (2-mercaptoethyl)sulfone (BMS) and N,N'-

bis(mercaptoacetyl)hydrazine (BMH) Claims - INNX -Confidential - a:\detailed description 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Elman's reagent, Dithitreitol (DTT), Dithioerythrithiol (DTE)

The present invention further also relates to an HCV NS3 protein as defined above which is additionally treated with a zwitterionic detergent, preferably Empigen. Other suitable detergents are known by the skilled man and are reviewed also in WO 96/04385.

The present invention further also relates to a method for purifying a cysteine containing recombinantly expressed protein comprising at least 2, preferably 3 or 4 and even more preferably all of the following steps:

(a) sulphonation of a lysate from recombinant host cells or lysis of recombinant host cells in the presence of guanidinium chloride (preferably 6 M Gu.HCl) followed by a subsequent sulphonation of the cell lysate,

(b) treatment with a zwitterionic detergent (preferably Empigen), preferably after removal of the cell debris,

(c) purification of the sulphonated version of the recombinant protein or purification of the sulphonated version of the recombinant protein with subsequent removal of the zwitterionic detergent, with said purification being preferably chromatography, more preferably a Ni-IMAC chromatography with said recombinant protein being a His-tagged recombinant protein,

(d) desulphonation of the sulphonated version of the recombinant protein, preferably with a molar excess of DTT,

(e) storage in the presence of a molar excess of DTT.

Empigen is also known as betaine and is a particularly preferred example of a zwitterionic detergent. The combination of such a detergent and DTT was found to be an ideal combination of agents to purify HCV NS3 helicase and HCV envelope proteins.

The present invention also relates to an HCV polynucleic acid encoding an HCV NS3 polyprotein or a unique part thereof as shown in Figure 1.

The present invention also relates to an HCV polynucleic acid as defined above characterized in Figure 2 (B9 clone) and by the fact that it does not react with false positive HCV samples, or a part thereof which encodes NS3 epitopes which do not react with false positive HCV samples. It was particularly surprising that the clone represented by SEQ ID NO has the property of ot reacting false positive HCV samples.

The present invention also relates to an HCV polynucleic acid as defined above characterized in Figure 2 (19B clone).

The present invention also relates to an HCV NS3 helicase protein as depicted in Figure 1 or an unique part thereof.

The present invention also relates to an HCV NS3 helicase protein or part thereof containing either A1218, A1384, P1407, V1412, P1424, or F1444, or a combination of amino acids S1200, L1201, A1218, S1222, I1274, S1289, T1321, A1323, T1369, L1382, A1384, P1407, V1408, G1409, F1410, V1412, P1424, F1444. Said numbering is according to the commonly accepted HCV amino acid numbering system.

The present invention also relates to any probes or primers derived from said polynuclic acids. The present invention also relates to recombinant vectors and hosts derived from said polynucleic acids. For detailed description to an overview of these applications reference is made to WO 96/13590.

The present invention also relates to an immunoassay comprising an HCV polypeptide as defined above. Said immunoassay can be of any type of format known in the art (see for instance WO 96/13590).

The present invention also relates to any method for producing and using said polyproteins of the invention. Methods for producing and using HCV polyproteins are disclosed in WO 96/13590. Said uses include not only diagnostic uses but also therapeutic and prophylactic uses. The NS3 proteins of the invention are also particularly suited to be incoporated in vaccine compositions. Said vaccine composition may contain besides the active ingredient any type of adjuvant known in the art. The contents of WO 96/13590 are hereby incorporated by reference in the present description. The NS3 proteins of the present invention may also be used in any application where it is applicable to use an NS3 helicase, such as for drug screening purposes.

EXAMPLES

Example 1. Expression of HCV NS-3 clones in *E. coli*

1.1 Cl ning of the HCCl19b cDNA fragment

The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from HCV subtype 1b serum IG8309 using synthetic oligonucleotide primers HCPr59 (5'-GGGCCCCACCATGGGGGTTGCGAAGGCGGTGGACTT-3') and HCPr60 (5'-CTATTAGCTGAAAGTCGACTGTCTGGGTGACAGCA-3').This yielded a PCR fragment 19 which was cloned into E. coli. The sense primer HCPr59 introduces an ApaI restriction site which includes an artifical methionine; antisense oligonucleotide HCPr60 introduces a stopcodon after aa 1465. The PCR fragment was subsequently cut with ApaI and the resulting 833 bp ApaI fragment was cloned in the ApaI cut IG expressionvector pmTNFHRP resulting in vector pmTNFHRPHCCl19b. Four hepatitis C clones (HCCl) were sequenced: HCCl19a, HCCl19b, HCCl19c, and HCCl19d (see deduced amino acid sequence given in Figure 1 and Figure 2).

1.2 Construction of the expression plasmid pEmTNFMPHHCCL-19b

Starting from vector pmTNFHRPHCCl19b the NS3 clone 19b coding sequence was isolated as a 900 bp NcoI fragment and inserted into the NcoI cut IG expressionvector pEmTNFMPH resulting in vector pEmTNFMPHHCCl19b. This plasmid expresses HCV NS3 clone 19b as an N-terminal fusionprotein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site.

1.3 Expression of HCV NS-3 clone 19b in *E.coli*

E. coli strain MC1061(pAcI) cells were transformed with plasmid pEmTNFMPHHCCl19b. MC1061(pAcI) cells harboring pEmTNFMPHHCCl19b were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD_{600} of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS-3 clone 19b fusion protein was analysed on western blot using specific monoclonal antibodies and HCV positive human sera.

Example 2. Expression f HCV NS3 cl ne B9 in *E.coli*

2.1 Cloning of the HCV NS-3 clone B9 gene

The NS3 helicase domain (amino acids 1188-1465) was amplified by RT-PCR from HCV subtype 1a serum IG21054 using synthetic oligonucleotide primers HCPr59 (5'-GGGCCCCACCATGGGGGTTGCGAAGGCGGTGGACTT-3') and HCPr60 (5'-CTATTAGCTGAAAGTCGACTGTCTGGGTGACAGCA-3').This yielded a PCR fragment B which was cloned into E. coli. The sense primer HCPr59 introduces an ApaI restriction site which includes an artifical methionine; antisense oligonucleotide HCPr60 introduces a stopcodon after aa 1465. The PCR fragment was subsequently cloned in the pGEM-T vector (Promega) and sequenced. Four clones were sequenced: B7, B9, B12, and B14 (see deduced amino acid sequences in Figure 1 and Figure 2). Clone B9 was retained for further subcloning.

2.2 Construction of the expressionplasmid pIGFH111NS-3B9

Starting from vector pGEMTNS3B9, the clone B9 coding sequence was isolated as a 850 bp NcoI/SpeI blunted fragment and inserted into the NcoI/StuI cut IG expression vector pIGFH111 resulting in vector pIGFH111NS3B9. This plasmid expresses HCV NS3 clone B9 as an N-terminal fusion protein with the N-terminal 25 aa of murine TNF followed by a hexahistidine purification tag and a formic acid cleavage site.

2.3 Expression of HCV NS-3 clone B9 in *E.coli*

E.coli strain MC1061(pAcI) cells were transformed with plasmid pIGFH111NS3B9. MC1061(pAcI) cells harboring pIGFH111NS3B9 were grown overnight in Luria Broth (LB) supplemented with 10 µg/ml tetracycline at 28°C. Cultures were diluted 20 times in fresh LB, then grown at 28°C to an OD_{600} of 0.2, after which the temperature was raised to 42°C. At 2 to 3 hours post-induction, the cells were harvested. Expression of the HCV NS3 clone B9 fusion protein was analysed on western blot using specific monoclonal antibodies and HCV positive

human sera.

Example 3. Expression of HCV NS3 clones A26, C16, and D18 in *E.coli*

Clones A26, C16, and D18 were isolated from HCV subtype 1a infected sera IG21051, IG17790, and 21068, respectively, in a similar way as described for clone B9 using primers HCPr59 and HCPr60. Initially, clones, A5, A26, C1, C3, C4, C12, C16, D17, D18, and D19, were cloned and sequenced. Clones A26, C16, and D18 were retained for further subcloning.

Example 4. Purification of the NS3 helicase protein domain

Nine volumes of 8M Gu.HCl and 1 volume of 0.2 M NaHPO were added to each gram equivalent of wet E. coli cell paste and the solution was homogenized by continuously vortexing. Solid $Na_2S_4O_6$ and Na_2SO_3 were added to the solution up to a final concentration of 65 and 360 mM respectively. $CuSO_4$ (stock solution: 0.1 M in 25% NH_3) was added up to a final concentration of 100µM.

The solution was stirred overnight in the dark at room temperature and after incubation at -70°C cleared by centrifugation at 4°C (30 min, 20.000 rpm, JA20 rotor).
Empigen BB ™ (Albright & Wilson) and imidazole were added to the supernatant up to a final concentration of 1% (w/v) and 20 mM respectively. the pH was adjusted to 7.2 with 1N HCl. A sample corresponding to 3 L cell culture equivalent was loaded at 2 mL/ min on a 25 mL Ni-IDA Sepharose FF (XK 16/20 column, Pharmacia), which had been equilibrated with buffer A containing 20 mM imidazole (buffer A: 50 mM phosphate, 6M Gu.HCl, 1% Empigen, pH 7.2). The Ni-IDA Sepharose column was washed consecutively with:

- buffer A containing 20mM imidazole
- buffer A containing 35 mM imidazole
- buffer A containing 50 mM imidazole
- buffer B containing 50 mM imidazole (buffer B: 50 mM Phosphate, 6M Gu.HCl, pH 7.2);
- buffer B containing 200 mM imidazole.

Each washing step was maintained during the chromatography untill the absorbance at 280 nm reached baseline level.

The column was regenerated with 50 mM EDTA, 500 mM NaCl, pH 7.0.

Fractions were analysed by SDS-PAGE under non reducing conditions and silver staining. The mTNF NS3 B9 fusion protein was recovered in the 200 mM imidazole elution.

Western blot with anti - hTNF (1µg NS3/ lane) and anti -E. coli (10 µgNS3/ lane) showed that the NS3 was over 99 % pure after this single chromatography step.

The 200 mM imidazole elution fractions were pooled and desalted.

A 40 mL Ni-IDA eluate sample was loaded at 10 mL /min on a 300 mL Sephadex G25 column (XK 50, Pharmacia)which had been equilibrated with 50 mM phosphate,6M ureum, 1mM EDTA, pH 7.2. 10 mL -fractions were collected and the protein concentration was determined by the micro BCA method (Pierce). The protein concentration was adjusted to 500 µg/mLwith the desalting buffer before desulphonation and reduction. The overall yield is 50-55 mg purified NS3 fusion protein/ L cell equivalent.

Finally, DTT (stock 100 mM in MQ water) was added in a 100 fold molar excess versus the cysteine content in the NS3 antigen (NS3= 7 cys).The solution was flushed with nitrogen and incubated for 1h at 28°C. The NS3 sample is diluted afterwards in the appropiate buffer for ELISA and LIA application.

Example 5. NS3 helicase antibody reactivity tested in LIA

In order to test the NS3 helicase antibody reactivity, a line of 50 µg/ml NS3 antigen solution in phosphate buffered saline was applied onto nylon membrane strips using a hamilton syringe. The strips were dried for at least 1 hour at a temperature between 18-24°C and were subsequently blocked with PBS/caseine in the presence (10 mM) or absence of the reducing agent DTT. The strips were subsequently washed with PBS containing Tween 20 and either no DTT or 10 mM DTT and with water containing either no DTT or 10 mM DTT and 1 mM EDTA. The membranes were dried for 30 minutes and cut into strips for testing of different patient samples.

The results of an experiment wherein strips were incubated with an anti-HCV seroconversion

panel (Boston Biomedica Inc.) are given in Table 1.

Example 6. NS3 helicase antibody reactivity tested in ELISA

In order to test the NS3 helicase antibody reactivity, ELISA plates were coated with the NS3 antigens purified as in Example 4 in the following way.

Microtiter plate wells were coated with NS3 protein at a concentration of 0.3 µg/ml NS3 protein in coating buffer containing 50 mM carbonate buffer, either 200 mM DTT or no DTT, and 1 mM EDTA. The microtiter plates are incubated for 18 hours at 20° C, and blocked with 300 µl of PBS/caseine buffer per well. The plates were incubated for 2 hours at 20°C and subsequently fixed with 300 µl of fixation buffer containing either 200 mM DTT or no DTT, and 1 mM EDTA for 2 hours at 20°C.

The results are shown in Tables 2 and 3. Table 2 gives the Signal to Noise values of assays including NS3 coated and fixed with or without DTT, with the BBI seroconversion panels PHV901 to PHV912. Table 3 shows a summary of the number of days in which HCV antibodies can be detected earlier by the assay incorporating DTT. Clearly, a total number of 34 days of earlier detection in 12 HCV seroconversions can be obtained by incorporating DTT in the assay.

Table 1: BBI panels tested in LIA coated with HCV NS3 as described in Example 4

PHV	+DTT	- DTT
903-01	-	-
903-02	-	-
903-03	+/-	-
903-04	2	-
903-05	2	+/-
903-06	2	+/-
903-07	4	2
903-08	4	2

Table 2: BBI panels tested in ELISA coated with HCV NS3 as described in Example 5

member ID #	bleed date	+DIT	-DIT
PHV901-01	09/23/93	0.1	0.3
PHV901-02	11/27/93	0.1	0.3
PHV901-03	12/29/93	2.0	2.9
PHV901-04	12/31/93	2.1	3.0
PHV901-05	01/05/94	2.2	3.1
PHV901-06	01/07/94	2.4	3.2
PHV901-07	02/01/94	4.1	6.0
PHV901-08	02/09/94	3.9	5.9
PHV901-09	03/01/94	4.0	7.9
PHV901-10	03/08/94	4.1	7.8
PHV901-11	04/14/94	4.2	8.3
PHV903-01	02/07/92	0.2	0.2
PHV903-02	02/12/92	0.9	0.9
PHV903-03	02/14/92	1.3	1.6
PHV903-04	02/19/92	2.5	2.7
PHV903-05	02/21/92	2.8	2.8
PHV903-06	02/26/92	3.2	4.6
PHV903-07	02/28/92	3.5	5.4
PHV903-08	03/04/92	3.5	4.1
PHV904-01	04/18/95	0.1	0.2
PHV904-02	04/20/95	0.1	0.3
PHV904-03	04/25/95	0.1	0.2
PHV904-04	04/27/95	0.1	0.2
PHV904-05	05/02/95	0.4	0.4
PHV904-06	05/09/95	0.8	0.5
PHV904-07	05/11/95	0.8	0.5
PHV905-01	11/17/95	0.1	0.2
PHV905-02	11/21/95	0.1	0.3
PHV905-03	11/24/95	0.1	0.3
PHV905-04	11/28/95	0.2	0.3
PHV905-05	12/01/95	0.5	0.3
PHV905-06	12/05/95	1.0	0.4
PHV905-07	12/08/95	2.5	0.8
PHV905-08	12/12/95	3.5	2.2
PHV905-09	12/15/95	3.5	3.2

member ID #	bleed date	+DIT	-DIT
PHV907-01	04/06/96	0.1	0.2
PHV907-02	04/10/96	0.1	0.2
PHV907-03	04/13/96	0.1	0.2
PHV907-04	04/19/96	3.0	2.2
PHV907-05	04/24/96	3.7	4.1
PHV907-06	04/27/96	3.6	4.1
PHV907-07	09/17/96	3.9	7.6
PHV908-01	01/28/96	0.1	0.1
PHV908-02	01/29/96	0.1	0.1
PHV908-03	01/31/96	0.1	0.1
PHV908-04	02/06/96	0.1	0.1
PHV908-05	02/08/96	0.1	0.1
PHV908-06	02/14/96	0.2	0.1
PHV908-07	02/20/96	1.4	0.2
PHV908-08	02/22/96	1.6	0.2
PHV908-09	02/27/96	1.9	0.2
PHV908-10	03/01/96	2.3	0.2
PHV908-11	03/07/96	2.3	0.4
PHV908-12	03/11/96	2.8	0.5
PHV908-13	03/14/96	2.8	0.5
PHV909-01	01/28/96	0.1	0.4
PHV909-02	02/15/96	2.3	5.4
PHV909-03	02/17/96	2.4	5.3
PHV910-01	08/28/96	0.1	0.2
PHV910-02	08/30/96	0.4	0.2
PHV910-03	09/03/96	2.7	3.1
PHV910-04	09/06/96	3.6	6.4
PHV910-05	09/10/96	3.9	8.1
PHV911-01	10/30/96	0.1	0.2
PHV911-02	11/02/96	0.1	0.2
PHV911-03	11/13/96	2.1	4.0
PHV911-04	11/20/96	3.6	7.8
PHV911-05	11/23/96	3.7	7.7
PHV912-01	01/06/96	0.2	0.3
PHV912-02	01/10/96	0.2	0.2
PHV912-03	01/13/96	4.5	9.9

member ID #	bleed date	+DIT	-DIT
PHV902-01	02/10/92	0.1	0.2
PHV902-02	02/12/92	0.1	0.2
PHV902-03	02/17/92	0.1	0.3
PHV902-04	02/19/92	0.3	0.6
PHV902-05	02/24/92	2.6	3.9
PHV902-06	02/26/92	3.1	5.9
PHV902-07	03/02/92	3.4	6.5
PHV906-01	10/07/95	0.5	0.3
PHV906-02	10/09/95	0.5	0.4
PHV906-03	10/14/95	1.8	0.6
PHV906-04	10/17/95	1.5	1.2
PHV906-05	10/21/95	2.2	3.0
PHV906-06	10/24/95	2.5	4.5
PHV906-07	10/26/95	2.9	5.7

Table 3: Overview of the BBI panels - number of days with earlier detection

PHV	+DTT	- DTT
901	0	0
902	0	0
903	0	0
904	0	0
905	7	0
906	3	0
907	0	0
908	24	0
910	0	0
911	0	0
912	0	0

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CLAIMS

1. Solid phase immunoassay comprising on said solid phase an antigen in the presence of a reducing agent.

2. Method for producing an immunoassay according to claim 1, wherein said reducing agent is added to said solid phase during the steps of coating, blocking and/or fixation of said antigen to said solid phase.

3. Method according to claim 2 wherein said reducing agent is added to said solid phase during the step of coating the antigen to the solid phase.

4. Method according to claim 2, wherein said reducing agent is added to said solid phase during the step of blocking said solid phase comprising the antigen applied thereto in the presence of a blocking agent such as caseine.

5. Method according to claim 2, wherein said reducing agent is added to said solid phase during the step of fixation of said solid phase comprising the antigen applied thereto in the presence of a fixating agent.

6. Method according to any of claims 1 to 5 wherein said reducing agent is DTT or DTE.

7. Method according to any of claims 1 to 5 wherein said reducing agent is used in a concentration range of 0.1 mM to 1 M, more particularly from 0.5 mM to500 mM, even more particular from 1 mM to 250 mM, some applications may require ranges from 0.5 to 50 mM, 1 to 30 mM, 2 to 20 mM, or 5 to 15 mM, or about 10 mM.

8. Method according to any of claims 2 to 7 wherein said antigen is an HCV NS3 protein.

9. Solid phase immunoassay produced by a method according to any of claims 2 to 8.

10. ELISA produced by a method according to claim 2 wherein said reducing agent is preferably added in the coating and fixation steps.

11. Line Immunoassay produced by a method according to claim 2 wherein said reducing agent is preferably added in the blocking step.

12. Use of an assay according to claims 9 to 11 for in vitro diagnosis of antibodies raised against an antigen as described in claim 1.

13. HCV NS3 protein treated by a method comprising the steps of sulphonation and subsequent desulphonation.

14. HCV NS3 protein according to claim 13 which is additionally treated with a zwitter-ionic detergent, preferably Empigen.

15. Method for purifying a cysteine containing recombinantly expressed protein comprising at least 2, preferably 3 or 4 and even more preferably all of the following steps:
(a) sulphonation of a lysate from recombinant host cells or lysis of recombinant host cells in the presence of guanidinium chloride followed by a subsequent sulphonation of the cell lysate,
(b) treatment with a zwitterionic detergent, preferably after removal of the cell debris,
(c) purification of the sulphonated version of the recombinant protein or purification of the sulphonated version of the recombinant protein with subsequent removal of the zwitterionic detergent, with said purification being preferably chromatography, more preferably a Ni-IMAC chromatography with said recombinant protein being a His-tagged recombinant protein,
(d) desulphonation of the sulphonated version of the recombinant protein, preferably with a molar excess of DTT,
(e) storage in the presence of a molar excess of DTT.

16. An HCV polynucleic acid encoding a polypeptide as depicted in Figure 1 or a unique part thereof, more particularly a polynucleic acid having a sequence as represented in Figure 2.

17. An HCV polynucleic acid according to claim 16 as depicetd in Figure 2 (B9 clone) and by the fact that it does not react with false positive HCV samples, or a part thereof which encodes NS3 epitopes which do not react with false positive HCV samples.

18. An HCV NS3 helicase protein or part thereof containing either A1218, A1384, P1407, V1412, P1424, or F1444, or a combination of amino acids S1200, L1201, A1218, S1222, I1274, S1289, T1321, A1323, T1369, L1382, A1384, P1407, V1408, G1409, F1410, V1412, P1424, F1444.

19. An HCV polypeptide encoded by a polynucleic acid according to claims 16 or 17.

Abstract

The present invention relates to a solid phase immunoassay comprising on said solid phase an antigen in the presence of a reducing agent. The present invention also relates to a method for purifying a cysteine containing recombinantly expressed protein comprising at least 2, preferably 3 or 4 and even more preferably all of the following steps:

(a) sulphonation of a lysate from recombinant host cells or lysis of recombinant host cells in the presence of guanidinium chloride followed by a subsequent sulphonation of the cell lysate,

(b) treatment with a zwitterionic detergent, preferably after removal of the cell debris,

(c) purification of the sulphonated version of the recombinant protein or purification of the sulphonated version of the recombinant protein with subsequent removal of the zwitterionic detergent, with said purification being preferably chromatography, more preferably a Ni-IMAC chromatography with said recombinant protein being a His-tagged recombinant protein,

(d) desulphonation of the sulphonated version of the recombinant protein, preferably with a molar excess of DTT,

(e) storage in the presence of a molar excess of DTT.

The present invention also relates to novel HCV NS3 sequences as depicted in Figures 1 and 2.

```
                1
NS3A5           MGVAKAVDFIPVENLETTMRSPVFTDNSSPPAVPQSFQVAHLHAPTGSGK
NS3A26           ---------------------------------------------------
NS3B7           ------------S-----------------------------------------
NS3B9           ------------S-----------------------------------------
NS3B12          ------------S-----------------------------------------
NS3B14          ------------S-----------------------------------------
NS3C1            ------------SM--------------------T-------------
NS3C3            ------------SM--------------------T------------S-
NS3C4            ------------SM--------------------T-------------
NS3C12           ------------SM--------------------T-------------
NS3C16           ------------SM--------------------T-------------
NS3D17           ---------------------------------------------------
NS3D18           ---------------------------------------------D---
NS3D19           ---------------------------------------------------
NS3FS13          ---------------------------------------------D---
NS3HCCL19A      --------V---SM--------------------T-------------
NS3HCCL19B      --------V---SM--------------------T-------------

                51
NS3A5           STKVPAAYAAQGFKVLVLNPSVAATLGFGAYMSRAHGIDPNIRTGVRTIT
NS3A26          --T---V-----------------------------------------------
NS3B7           ---------G--Y--------------L-----KV---------------
NS3B9           ------------Y--------------------K----------------
NS3B12          ------------Y--------------------K----------------
NS3B14          ------------Y-M------------------K-Y--------------
NS3C1           ------------Y--------------------K------G---------
NS3C3           ------------Y--------------------K------G---------
NS3C4           ------------Y--------------------K------G---------
NS3C12          ------------Y--------------------K------G---------
NS3C16          ------------Y--------------------K------G---------
NS3D17          ------------Y--------------------K---V------------
NS3D18          ------------Y--------------------K---V------------
NS3D19          ------------Y--------------------K---V------------
NS3FS13         ------------Y--------------------K---V------------
NS3HCCL19A      ------------Y--------------------K---V------------
NS3HCCL19B      ------------Y--------------------K---V------------
```

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Figure 1-2

```
                101
NS3A5           TGSPITYSTYGKFLADGGCSGGAYDIMICDECHSTDATSILGIGTVLDQA
NS3A26          ----------------------------I----------------------
NS3B7           ----------------------------I----------------------
NS3B9           ----------------------------I----------------------
NS3B12          ----------------------------I-------------D--------
NS3B14          ----T-------------R-------I-----------------------
NS3C1           --A-------------------------I---------S-T-----------
NS3C3           --A-------------------------I---------S-T-----------
NS3C4           --A-------------------------I---------S-T-----------
NS3C12          --A-------------------------I---------S-T-----------
NS3C16          --A-------------------------I---------S-T-----------
NS3D17          ----------------------------I---------V-------------
NS3D18          ---------------N----------I---------V-------------
NS3D19          ----------------------------I---------V-------------
NS3FS13         ---------------N----------I---------V-------------
NS3HCCL19A      --A-------------------------I-------I-S-------------
NS3HCCL19B      --A-------------------------I-------I-S-------------

                151
NS3A5           ETAGARLVVLATAAPPGSVTVPHPNIEEVALSTTGEIPFYGKAIPLEAIK
NS3A26          -------------T-----------------------------------------
NS3B7           -------------T--------------------------C-----------
NS3B9           -------------T-----------------------------------------
NS3B12          -------------T-----------------------------------------
NS3B14          -------------T-----------------------------------------
NS3C1           -------------T-------------------N--------------T--
NS3C3           -------------T-------------------N--------------T--
NS3C4           -------------T-------------------N--------------T--
NS3C12          -------------T-------------------N--------------T--
NS3C16          -------------T-------------------N--------------T--
NS3D17          -------------T-----------------------------------------
NS3D18          -------------T-----------------------------------------
NS3D19          -------------T-----------------------------------------
NS3FS13         -------------T-----------------------------------------
NS3HCCL19A      -------------T-------------------SI-----------I-V--
NS3HCCL19B      -------------T-------------------S------------I-V--
```

```
                201
NS3A5           GGRHLIFCHSKKKCDELAAKLTALGVNAVAYYRGLDVSVIPTSGDVVVVA
NS3A26          ---R----------------------------------------------
NS3B7           ------------------N-V-----P----------------------
NS3B9           --------------------PV---------------P------------
NS3B12          --------------------V------------------------------
NS3B14          -----V---------------V------------------------------
NS3C1           --------------------SS--L--------------------------
NS3C3           --------------------SS--L--------------------------
NS3C4           --------------------SS--L--------------------------
NS3C12          --------------------SS--L--------------------------
NS3C16          --------------------SS--L--------------------------
NS3D17          ---------------K-----V---I--------------------------
NS3D18          --------------------V---I--------------------------
NS3D19          --------------------QV---I--------------------------
NS3FS13         --------------------V---I--------------------------
NS3HCCL19A      --------------------SGV-I--------------------------
NS3HCCL19B      --------------------SGF-I--------------------------

                251
NS3A5           TDALMTGYTGDFDSVIDCNTCVTQTVDFS
NS3A26          -----------------------------
NS3B7           -----------------------------
NS3B9           -------F---------------------
NS3B12          -------F---------------------
NS3B14          -------F--------------------R
NS3C1           -------F-------------A-------
NS3C3           -------F-------------A-------
NS3C4           -------F---------------------
NS3C12          -------F---------------------
NS3C16          -------F---------------------
NS3D17          ----------------------------I-
NS3D18          -----------------------------
NS3D19          -----------------------------
NS3FS13         -----------------------------LDPTFTIETTTLPQDAVSRTQ
NS3HCCL19A      -------F---------------------
NS3HCCL19B      -------F---------------------

                301
NS3FS13         RRGRTGRGKPGIYRFVTPGERPSGMFDSSVLCECYDAGCAWYELTPAETT
                351
NS3FS13         VRLRAYMNTPGLPVCQDHLEFWEGVFTGLTHIDAHFLSQTKQSGENLPYL
                401
NS3FS13         VAYQATVCARAQAPPPSWDQMWKCLIRLKPTLHGPTPLLYRLGAVQNEVT
                451
NS3FS13         LTHPVTKCIMTCMSADLQVVT
```

Figure 2 – 1

DNA coding sequence of the mTNFH6NS-3 clone 19b fusion protein:

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTC
GTAGCAAACCACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCAC
GTGGATCCCGGGCCCATGGGGGTTGCGAAGGCGGTGGACTTTGTACCCGTAG
AGTCTATGGAAACCACCATGCGGTCCCCGGTCTTTACGGATAACTCATCTCCTCC
GGCCGTACCGCAGACATTCCAAGTGGCCCATCTACACGCCCCCACTGGTAGTGG
CAAGAGCACTAAGGTGCCGGCTGCATATGCAGCCCAAGGGTACAAGGTACTTGT
CCTGAACCCATCCGTTGCCGCCACCTTAGGATTCGGGGCGTATATGTCTAAAGC
ACATGGTGTCGACCCTAACATTAGAACTGGGGTAAGGACCATCACCACGGGCGC
CCCCATTACGTACTCCACCTACGGCAAGTTTCTTGCCGACGGTGGTTGCTCTGGG
GGCGCTTACGACATCATAATATGTGATGAGTGCCACTCGATTGACTCAACCTCC
ATCTTGGGCATCGGCACCGTCCTGGATCAGGCGGAGACGGCTGGAGCGCGGCTT
GTCGTGCTCGCCACTGCTACACCTCCGGGGTCGGTCACCGTGCCACATCCCAAC
ATCGAGGAGGTGGCTCTGTCCAGCACTGGAGAGATCCCCTTTTATGGCAAAGCC
ATCCCCATCGAGGTCATCAAAGGGGGGAGGCACCTCATTTTCTGCCATTCCAAG
AAGAAATGTGACGAGCTCGCCGCAAAGCTATCGGGCTTCGGAATCAACGCTGTA
GCGTATTACCGAGGCCTTGATGTGTCCGTCATACCGACTAGCGGAGACGTCGTT
GTTGTGGCAACAGACGCTCTAATGACGGGCTTTACCGGCGACTTTGACTCAGTG
ATCGACTGTAACACATGCGTCACCCAGACAGTCGACTTCAGCTAA

Sequence depicted in **bold** is non-NS-3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

Figure 2-2

Amino Acid sequence of the mTNFH6NS-3 clone 19b fusi nprotein:

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMGVAKAVDFVPVES
METTMRSPVFTDNSSPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNP
SVAATLGFGAYMSKAHGVDPNIRTGVRTITTGAPITYSTYGKFLADGGCSGGAYDII
CDECHSIDSTSILGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEVALSSTGEIP
FYGKAIPIEVIKGGRHLIFCHSKKKCDELAAKLSGFGINAVAYYRGLDVSVIPTSGDV
VVVATDALMTGFTGDFDSVIDCNTCVTQTVDFS

Sequence depicted in **bold** is non-NS-3 sequence. This sequence contains the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

Figure 2 - 3

DNA coding sequence of the mTNFH6NS-3 clone B9 fusionprotein:

ATGGTAAGATCAAGTAGTCAAAATTCGAGTGACAAGCCTGTAGCCCACGTC
GTAGCAAACCACCAAGTGGAGGAGCAGGGAATTCACCATCACCATCACCAC
GTGGATCCCGGGCCCATGGGGGTTGCGAAGGCGGTGGACTTTATCCCCGTGGA
GAGCCTAGAAACAACCATGAGGTCCCCGGTGTTCACAGACAACTCCTCCCCGCC
AGCAGTGCCCCAGAGCTTCCAGGTGGCCCACCTGCATGCTCCCACCGGCAGCGG
TAAGAGCACCAAGGTCCCGGCCGCATATGCGGCTCAGGGCTACAAAGTGCTGGT
GCTCAACCCCTCCGTTGCTGCAACATTGGGCTTTGGTGCTTACATGTCCAAGGCC
CATGGGATTGATCCTAACATCAGGACTGGGGTAAGGACAATTACTACTGGCAGC
CCCATCACGTACTCCACCTACGGCAAGTTCCTTGCCGACGGCGGGTGCTCGGGGG
GTGCTTATGACATAATAATTTGTGACGAGTGCCACTCCACAGATGCAACATCTAT
TTTGGGCATCGGCACTGTCCTTGACCAAGCAGAGACTGCGGGGGCGAGACTGGT
TGTGCTTGCCACCGCTACCCCTCCGGGCTCCGTCACTGTGCCCCATCCTAATATCG
AGGAGGTTGCTCTGTCCACCACCGGAGAGATCCCCTTTTACGGCAAGGCTATCCC
CCTTGAGGCAATCAAAGGGGGGAGACATCTCATCTTCTGCCACTCAAAGAAGAA
GTGCGACGAACTCGCCGCCAAACCGGTCGCGTTGGGTGTCAATGCCGTGGCTTAC
TACCGCGGCCTTGACGTGCCCGTCATCCCGACCAGTGGCGATGTTGTCGTCGTGG
CAACTGATGCTCTCATGACCGGTTTTACCGGTGACTTCGACTCGGTGATAGACTG
TAATACGTGTGTCACCCAGACAGTCGACTTCAGCTAA

Sequence depicted in **bold** is non-NS-3 sequence. This sequence encodes the mTNF fusionpartner, the hexahistidine tag and part of the multilinker.

Amino Acid sequence of the mTNFH6NS-3 clone B9 fusionprotein:

MVRSSSQNSSDKPVAHVVANHQVEEQGIHHHHHHHVDPGPMGVAKAVDFIPVESL
ETTMRSPVFTDNSSPPAVPQSFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSV
AATLGFGAYMSKAHGIDPNIRTGVRTITTGSPITYSTYGKFLADGGCSGGAYDIIICDE
CHSTDATSILGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEEVALSTTGEIPFY

Figure 2-4

GKAIPLEAIKGGRHLIFCHSKKKCDELAAKPVALGVNAVAYYRGLDVPVIPTSGDVV
VVATDALMTGFTGDFDSVIDCNTCVTQTVDFS